High Degree of Similarity between *Chromatium vinosum* and *Chromatium minutissimum* as Revealed by Riboprinting

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The riboprinting technique (restriction fragment length polymorphism [RFLP] analysis of PCR-amplified ribosomal DNA) was used to study five strains representing three species of the genus *Chromatium*. An RFLP analysis following digestion of the amplified small-subunit ribosomal DNA with 25 restriction enzymes revealed that the patterns obtained for all strains of *Chromatium vinosum* were identical. *Chromatium gracile* was different from *C. vinosum* with seven enzymes. On the other hand, *Chromatium minutissimum* produced the same patterns as *C. vinosum* with all enzymes, indicating that these organisms have a high degree of similarity. An RFLP analysis of the PCR-amplified spacer sequence between the 16S and 23S ribosomal DNAs gave similar results except that there was a larger number of differences between *C. gracile* and the other organisms examined.

The arrangement of genera and species in the family *Chromatiaceae* (the purple sulfur bacteria) is based entirely on simple phenotypic characteristics. The criteria used to arrange these organisms in *Bergey's Manual of Systematic Bacteriology* (14) are the same as the criteria used in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (13).

The genus *Chromatium* is separated from other genera of motile purple sulfur bacteria with intracellular sulfur globules by its motility, its rod-shaped morphology, and its lack of gas vacuoles. Differentiation of species within the genus *Chromatium* is based entirely on phenotypic characteristics (e.g., the size of cells and the color of cultures). The DNA base ratios of members of the genus (48.0 to 70.4 mol% G+C) indicate that genetically distantly related species are grouped together. At the species level, broad ranges of G+C contents also show that some species (e.g., *Chromatium vinosum* [G+C content range, 61 to 66 mol%]) may include heterogeneous collections of only phenotypically similar strains.

The genus *Chromatium* consists of two distinct groups based on cell size; the large *Chromatium* spp. include *Chromatium okenii*, *Chromatium weissei*, *Chromatium warmingii*, and *Chromatium buderi*, while the small *Chromatium* spp. include *Chromatium minus*, *C. vinosum*, *Chromatium violascens*, *Chromatium gracile*, and *Chromatium minutissimum* (14).

In the group containing the small *Chromatium* spp. there are no characteristics that separate *C. minutissimum* and *C. vinosum* other than a difference in cell size. *C. minutissimum* is differentiated by its very small cell size (1 to 1.2 μm wide by 2 μm long) and its rod-shaped cells. All other characteristics are the same as the characteristics of *C. vinosum*. Moreover, the G+C content of *C. minutissimum* (63.7 mol%) falls within the range given for *C. vinosum* strains (61.3 to 66.3 mol%). On the other hand, the only phenotypic difference between *C. gracile* (rod-shaped cells that are 1 to 1.3 μm wide and 2 to 6 μm long) and *C. vinosum* is the requirement for 2 to 3% (wt/vol) NaCl for growth of the former. Most of the other characteristics of *C. gracile* are the same as *C. vinosum* characteristics. In addition, *C. gracile* can use some organic compounds, such as lac-

tate, propionate, and butyrate (11). The G+C content range of *C. gracile*, 68.9 to 70.6 mol%, is higher than the G+C content range given for *C. vinosum*. The two other species in the group containing the small *Chromatium* spp. differ in more characteristics, and we included in this study only the three *Chromatium* spp. that are very similar. Phylogenetic relationships were established in the family *Chromatiaceae* by Fowler et al. (9), who included *C. vinosum* but not *C. minutissimum* or *C. gracile* in their study.

There have been extensive reports on the use of ribotyping for identification of bacteria. However, this technique is time consuming and labor intensive as hybridization procedures and, in most cases, radioactive detection are required. Therefore, we used riboprinting instead (3). In riboprinting the highly conserved small-subunit (SSU) ribosomal DNA (rDNA) is amplified by PCR, the PCR product is cut with restriction enzymes, and the resulting reaction products are separated in an agarose gel and stained with ethidium bromide. There is no need for hybridization or radioactivity when this technique is used. Riboprinting has been used with much success to distinguish species belonging to different protist genera (2, 4, 6). Amplification of the more diverse intergenic (16S-23S) spacer region has been used frequently for the identification of bacteria. After amplification the spacer region is sequenced (10, 17) or hybridized with a specific probe (1). The numbers and sizes of the bands obtained after amplification of the spacer region have been shown to be diagnostic for species (8, 12). In this study we used these techniques to study the relationships of three *Chromatium* species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. vinosum* ATCC 17899, DSM 180, and DSM 185, *C. minutissimum* DSM 1376, and *C. gracile* DSM 203 were used in this study. All five strains were obtained from I. Esteve's laboratory at the Autonomous University of Barcelona, Barcelona, Spain.

Cultures were grown photolithoautotrophically in 125-ml rubber-capped bottles at 23°C with 50 microeinsteins of continuous illumination $\cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Liquid Pfennig minimal medium (18) was used to obtain a cell concentration of about 10^8 cells ml^{-1} . The axenic condition of the cultures was determined by microscopic observation.

Preparation of biological material. Cells from 25-ml portions of light-grown cultures of the five strains were collected by centrifugation, resuspended in 1.5 ml of SE buffer (100 mM EDTA, 100 mM NaCl, 10 mM Tris [pH 7.8]), and transferred to Eppendorf tubes. Sodium dodecyl sulfate (0.1%) and proteinase K (50 $\mu\text{g}/\text{ml}$) were added to each tube, and each mixture was incubated at 37°C for 1 h. The suspensions were treated with phenol-chloroform-isoamyl alcohol (50:

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FIG. 1. RFLP analysis of the PCR-amplified SSU rDNAs (ribotyping) of the five *Chromatium* strains studied. *C. gracile* had a different riboprint than the other four strains with restriction enzymes *AluI*, *AvaII*, *BglII*, *DdeI*, *HhaI*, *MboI*, and *PstI*. *C. minutissimum* and the three strains of *C. vinosum* had the same riboprint.

FIG. 2. RFLP analysis of the PCR-amplified 16S-23S ITS (ribotyping) of the five *Chromatium* strains studied. *C. gracile* had a different riboprint than the other four strains with restriction enzymes *DdeI*, *NciI*, *MboI*, *MspI*, *AvaII*, *HindIII*, *PstI*, *SstI*, *AluI*, *AvaI*, *BanI*, *HinfI*, and *HaeIII*. *C. minutissimum* and the three strains of *C. vinosum* had the same riboprint.

TABLE 1. RFLP analysis of the PCR-amplified SSU rRNA gene and ITS of the 16S-23S genes of *C. vinosum*, *C. minutissimum*, and *C. gracile*

Restriction enzyme	Results of RFLP analysis ^a	
	SSU rDNA	ITS
<i>AccI</i>	NC	NC
<i>AluI</i>	DIF	DIF
<i>ApaI</i>	ID	NC
<i>AvaI</i>	ID	DIF
<i>AvaII</i>	DIF	DIF
<i>BamHI</i>	NC	NC
<i>BanI</i>	ID	DIF
<i>BglII</i>	DIF	NC
<i>DdeI</i>	DIF	DIF
<i>EcoRI</i>	ID	NC
<i>HaeIII</i>	ID	DIF
<i>HhaI</i>	DIF	ID
<i>HincII</i>	ID	NC
<i>HindIII</i>	NC	DIF
<i>HinfI</i>	ID	DIF
<i>KpnI</i>	NC	NC
<i>MboI</i>	DIF	DIF
<i>MboII</i>	NC	NC
<i>MspI</i>	ID	DIF
<i>NciI</i>	ID	DIF
<i>PstI</i>	DIF	DIF
<i>RsaI</i>	ID	NC
<i>SalI</i>	ID	NC
<i>SmaI</i>	ID	NC
<i>SstI</i>	NC	DIF

^a NC, the enzyme did not cut the DNA of any strain; ID, the patterns obtained for all strains were identical; DIF, the pattern obtained for *C. gracile* was different from the other patterns.

49:1), and the DNAs were precipitated with ethanol. RNA was eliminated by treatment with RNase (20 µg/ml) for 30 min at 37°C. The extracted DNA was dissolved in 500 µl of distilled water and stored at -20°C until it was needed.

PCR amplification. PCR amplification and purification of the products were performed essentially as described previously (3) by using *Taq* DNA polymerase obtained from Boehringer, Mannheim, Germany. The annealing temperature was increased to 58°C for SSU rDNA amplification to eliminate some additional small bands obtained at 55°C. The following primers used to amplify the gene encoding SSU rRNA were designed by comparing conserved sequences at the 5' and 3' ends of previously described SSU rDNAs of purple bacteria, including *C. vinosum* (GenBank accession number M26629): forward primer 5'-AGAGTTTG ATCCTGGCTCAG3' and reverse primer 5'-TGGTGTGACGGGCGGTGT GT3'. Two other primers were used to amplify the spacer region between the 16S and 23S rRNA genes; forward primer 5'-ACACACGCCCCGTACACCA3' was complementary to the 3' end of the SSU primer, and reverse primer 5'-GCCAA GGCATCCACC3' was designed by comparing the 5' ends of previously described bacterial large-subunit rDNA sequences. The primers were constructed by Pharmacia, Uppsala, Sweden.

In the initial analysis the PCR products were electrophoresed in 0.7% (wt/vol) agarose minigels. The oil overlay in the PCR tube was eliminated by extraction with chloroform. The DNA was precipitated with ethanol and dissolved in 100 µl of distilled water.

Restriction fragment length polymorphism (RFLP) analysis. The amplified SSU rDNA and spacer region DNA were treated for 1 h at 37°C with 25 restriction enzymes by using the specifications of the supplier (Life Technologies, Inc., Gaithersburg, Md.). The restriction enzymes tested were *AccI*, *AluI*, *ApaI*, *AvaI*, *AvaII*, *BamHI*, *BanI*, *BglII*, *DdeI*, *EcoRI*, *HaeIII*, *HhaI*, *HincII*, *HindIII*, *HinfI*, *KpnI*, *MboI*, *MboII*, *MspI*, *NciI*, *PstI*, *RsaI*, *SalI*, *SmaI*, and *SstI*.

The digested DNA was separated in 2% (wt/vol) agarose gels together with a 123-bp DNA ladder marker (Life Technologies, Inc.) and stained with ethidium bromide.

RESULTS

SSU rDNA. The sizes of the amplified SSU rDNA products of all of the *Chromatium* strains investigated were the same.

Six restriction enzymes, *AccI*, *BamHI*, *HindIII*, *KpnI*, *MboII*, and *SstI*, did not cut any of the DNA preparations.

Nineteen restriction enzymes, *AluI*, *ApaI*, *AvaI*, *AvaII*, *BanI*, *BglII*, *DdeI*, *EcoRI*, *HaeIII*, *HhaI*, *HincII*, *HinfI*, *MboI*, *MspI*, *NciI*, *PstI*, *SalI*, *SmaI*, and *RsaI*, generated identical patterns for *C. minutissimum* and the three strains of *C. vinosum* studied. The lengths of the fragments were the lengths expected from the previously reported sequence of *C. vinosum* (GenBank accession number M26629).

A total of 7 of the 25 restriction enzymes, *AluI*, *AvaII*, *BglII*, *DdeI*, *HhaI*, *MboI*, and *PstI*, revealed differences between *C. gracile* and the four other strains studied (Fig. 1).

Spacer region. The amplified spacer region was approximately 600 bp long in *C. gracile* and almost 700 bp long in the other *Chromatium* strains. In *C. gracile*, three additional fainter bands larger than the main band were observed. In the four other strains, a fainter band smaller than the main band was observed.

Eleven restriction enzymes, *AccI*, *ApaI*, *BamHI*, *BglII*, *EcoRI*, *HincII*, *KpnI*, *MboII*, *RsaI*, *SalI*, and *SmaI*, did not cut any of the DNA preparations.

Fourteen restriction enzymes, *AluI*, *AvaI*, *AvaII*, *BanI*, *DdeI*, *HaeIII*, *HhaI*, *HindIII*, *HinfI*, *MboI*, *MspI*, *NciI*, *PstI*, and *SstI*, generated identical patterns for *C. minutissimum* and the three strains of *C. vinosum*.

A total of 13 of the 25 restriction enzymes, *AluI*, *AvaI*, *AvaII*, *BanI*, *DdeI*, *HaeIII*, *HindIII*, *HinfI*, *MboI*, *MstI*, *NciI*, *PstI*, and *SstI*, revealed differences between *C. gracile* and the four other strains (Fig. 2). The data obtained in this study are summarized in Table 1.

DISCUSSION

On the basis of the identical riboprints we concluded that *C. minutissimum* is very similar to *C. vinosum*. Since only one strain of *C. minutissimum* was used and only one method was used, no conclusion concerning the relationship of these organisms can be drawn from the results of our analyses. Riboprinting is a powerful technique for clarifying the real identities of microorganisms whose morphological differences are minimal. This technique revealed that the pathogenic and non-pathogenic forms of the intestinal parasite genus *Entamoeba* are in fact two different species (5). A free-living amoeba that on morphological grounds was described as a *Willaertia* sp. turned out to be a *Naegleria* sp. with an aberrant life cycle when riboprinting was used (7).

In some bacterial groups in which strain isolation and cultivation are not easy, phenotypic information may not be sufficient for classification. This is the case with phototrophic bacteria. Genotypic information may help in these cases to provide greater insight into the taxonomy of the groups. Although different physiological and ecological aspects of the purple phototrophic bacteria have been described, little is known about the genetics of these organisms. Our knowledge of anoxyphotobacteria is confined to the family *Rhodospirillaceae* (15, 16). Riboprinting may be used as a fast and cost-effective molecular technique to help clarify the taxonomy of special bacterial groups.

As expected, the SSU rRNA gene of *Chromatium* spp. is more conserved than the intergenic spacer region (internally transcribed spacer [ITS]) between the 16S and 23S ribosomal genes. In this study 7 and 13 of 25 restriction enzymes revealed RFLP differences between *C. vinosum* and *C. gracile* in the SSU and ITS regions, respectively. Identical RFLP patterns for the SSU and ITS regions were obtained for the three *C. vinosum* strains and the *C. minutissimum* strain. The amplified ITS of these strains were also the same size. We concluded that *C. minutissimum* is very similar to *C. vinosum*.

ACKNOWLEDGMENTS

We thank Vicky Pavón, Núria Gaju, and Isabel Esteve of the Institute of Fundamental Biology at the Autonomous University of Barcelona for providing the *Chromatium* cultures.

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